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Seven Transmembrane-spanning Receptors for Free Fatty Acids as Therapeutic Targets for Diabetes Mellitus: Pharmacological, Phylogenetic, and Drug Discovery Aspects*

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Free fatty acids (FFAs)[2] take part in many physiological processes in different tissues such as skeletal muscle, liver, heart, and pancreas by providing an oxidative energy source. In addition, FFAs are potent signaling molecules (1). Dysregulation of FFA metabolism is responsible for insulin resistance and type 2 diabetes mellitus (2). The presence of some FFAs is essential for glucose-stimulated insulin secretion from pancreatic β-cells. However, if FFAs are chronically in excess, they can reduce insulin biosynthesis and secretion and induce β-cell apoptosis (2). The regulatory effect of FFAs occurs in part by their involvement as substrates in intracellular lipid signaling pathways; however, FFAs also signal directly via seven transmembrane-spanning receptors (7TMRs; G protein-coupled receptors). Here, we consider 7TMRs that are activated by FFAs and FFA amides. Furthermore, we describe the identification and characterization of small molecule ligands for these FFA receptors (FFARs) that may be useful for treating patients with diabetes mellitus.

Free Fatty Acid Receptors

All members of a previously characterized cluster of orphan 7TMRs comprising FFAR1 (previously known as GPR40), FFAR2 (previously known as GPR43), and FFAR3 (previously known as GPR41) are activated by FFAs within physiological concentration ranges (3). More recently, the orphan receptors GPR119 and GPR120 were identified as targets of FFAs and FFA amides, respectively (4, 5). The FFAR1 cluster is tandemly located on chromosome 19q13.1. These receptors share a relatively high sequence similarity but have diverse tissue distribution. FFAR1 and GPR120 are activated by medium- to long-chain fatty acids, whereas FFAR2 and FFAR3 are activated by short-chain fatty acids. GPR119 is activated by long-chain FFA amides such as oleoylethanolamide and lysophosphatidylcholine (6, 7).

FFAR1 couples preferentially to G_q to stimulate the activity of phospholipase C (8, 9). FFAR3 appears to be selective for G_i activation, whereas FFAR2 can activate G_i and G_q (3). GPR119 is a G_s-coupled receptor (10). The ability of FFAs to elevate Ca^{2+} in mouse intestinal endocrine STC-1 cells via GPR120 indicates interaction with members of the G_q family (11).

FFAR1 is highly expressed in the insulin-secreting β-cells of the pancreas (8) and is present at lower levels in pancreatic α-cells also (12). GPR119 is significantly enriched in human pancreatic and gastrointestinal tissue (13); it is expressed mainly in β-cells of isolated pancreatic islets from mice (14). GPR120 is abundantly expressed in the intestine but is not expressed in the pancreas or clonal β-cells (5). FFAR2 and FFAR3 have different tissue distributions and more broad expression profiles; FFAR2 is abundant in leukocytes and adipose tissue (6), whereas FFAR3 is highly expressed in brain, lung, and adipose tissue.

Sequence and Phylogenetic Analysis

7TMRs form a large transmembrane protein superfamily that encompasses >1000 unique members in the human genome. They are composed of a single polypeptide chain that crosses the plasma membrane seven times via α-helical transmembrane domains (TMs). The N-terminal ends of these receptors are located extracellularly, whereas their C-terminal ends are in the cytoplasm. Phylogenetic analyses show that 7TMRs are clustered into five families: glutamate, rhodopsin, adhesion, Frizzled/Taste2, and secretin (15). The four human 7TMRs for FFAs and the single human receptor for FFA amides (6, 16) belong to the rhodopsin family, a tree representation of which is provided in Fig. 1.

According to sequence similarity, FFAR1, FFAR2, and FFAR3 form a well defined receptor cluster (the FFAR1 cluster) that belongs to the subfamily of nucleotide and lipid receptors (17). The TMs of the three receptors share ~39% sequence identity. FFAR1 is the most dissimilar receptor of the cluster, sharing ~35% TM sequence identity with other members. As mentioned above, FFAR1 is the only receptor in the cluster to be selective for medium- to long-chain fatty acids, whereas FFAR2 and FFAR3 are activated by short-chain fatty acids.

Receptors of the nucleotide and lipid subfamily are typically activated by negatively charged ligands and are characterized by the presence of basic residues at specific positions within their TMs. A chemogenomic analysis of 7TMRs, conducted comparing 30 residues within the putative common binding cavity located in the TM bundle of the receptors, suggested the involvement of two conserved Arg residues at positions 5.39 and 7.35 in the coordination of the negative charges of FFAs by the receptors belonging to the FFAR1 cluster (18).[3] This analysis also proposed the difference in bulkiness of the residue at

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[2] The abbreviations used are: FFAs, free fatty acids; 7TMRs, seven transmembrane-spanning receptors; FFARs, FFA receptors; TMs, transmembrane domains.

[3] TM positions are identified with the indexing scheme of Ballesteros and Weinstein (19).

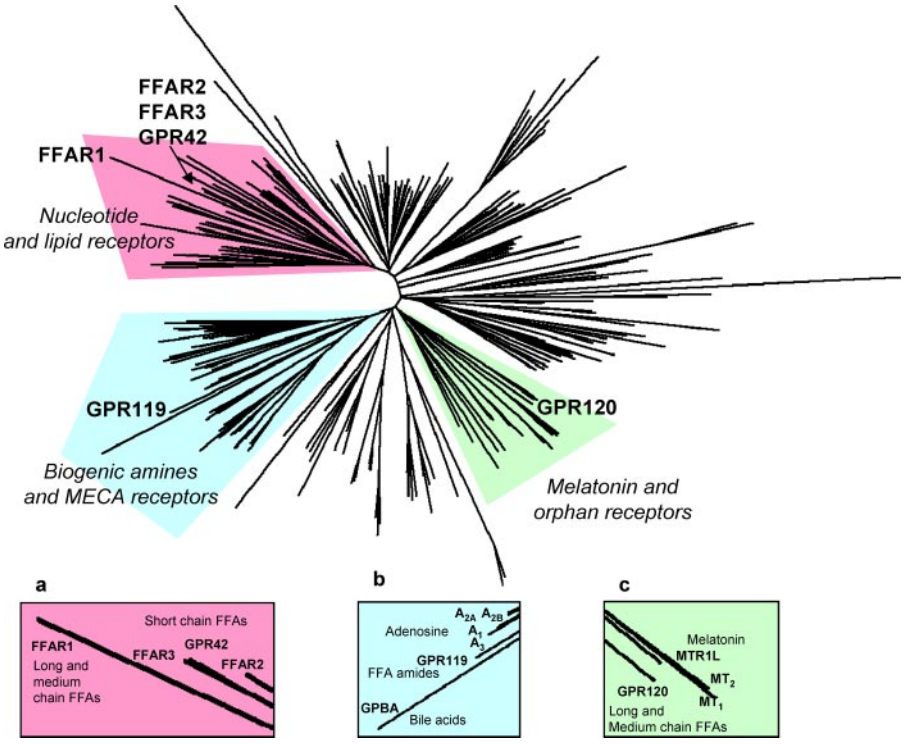

FIGURE 1. **Phylogenetic tree of the rhodopsin family of 7TMRs.** FFAR1, FFAR2, and FFAR3, which are all activated by FFAs, form a receptor cluster that belongs to the nucleotide and lipid receptor subfamily (*pink*). *GPR42* (not discussed in this review) is most likely a pseudogene resulting from the duplication of the *FFAR3* gene, with which it shares 99% sequence identity in the TMs. GPR120, which is also a receptor for FFAs, belongs to a subfamily that encompasses receptors for melatonin as well as a number of orphan receptors (*green*). GPR119, which is a receptor for FFA amides, belongs to the subfamily of biogenic amine and MECA receptors (*turquoise*). Close-ups of the receptors for FFAs and FFA amides and their nearest neighbors are provided in *insets a–c*. Detailed trees for the families of nucleotide and lipid receptors and of biogenic amines and MECA receptors have been published elsewhere (17, 21, 25). *MTs*, receptors for melatonin; *GPBA*, receptor for bile acids.

position 6.48, located deeply in the binding cavity, as an explanation for the selectivity of long- *versus* short-chain fatty acids. Site-directed mutagenesis experiments, guided by molecular modeling, did not confirm the latter hypothesis but did confirm the proposed role of the conserved Arg residues (17).

The medium- to long-chain FFAR GPR120 does not belong to the FFAR1 cluster. Although evolutionarily conserved, GPR120 lacks close relatives (20) and belongs to a subfamily that includes several orphan receptors and a cluster of melatonin receptors. Although GPR120 and FFAR1 are both activated by long-chain FFAs, the two receptors exhibit only 19% sequence identity. Moreover, our models predict that they do not share ligand recognition determinants: none of the FFAR1 residues proposed to be involved in ligand binding are conserved in GPR120, and this receptor does not have basic residues in the TM cavity.

As mentioned above, long-chain FFA amides such as oleoylethanolamide have been identified as the endogenous ligands of GPR119. This receptor is also activated by phospholipids and belongs to the subfamily of the biogenic amine and MECA (melanocortin, endothelial, cannabinoid, and adenosine) receptors, a tree representation of which is provided elsewhere (21). GPR119 does not show significant similarity to the known receptors for FFAs, sharing only 24 and 18% TM sequence identity with GPR120 and FFAR1, respectively. The different clustering of FFAR1 and GPR119 based on sequence comparison is in line with the differences in their ligand selectivity: FFAR1 and relatives are activated by FFAs and cluster with receptors typically activated by negatively charged ligands such as nucleotide and dicarboxylic acids, whereas GPR119 is activated by FFA amides and clusters with receptors typically for neutral ligands such as nucleosides. The closest homologs of GPR119 are the adenosine A_1 and A_3 receptors, with which it shares 28% TM sequence identity.

Homology Model of FFAR1

Molecular determinants for the recognition of linoleate and GW9508 (a synthetic agonist) (22, 23) by FFAR1 were proposed on the basis of sequence comparisons, rhodopsin-based homology modeling, and mutagenesis (17, 24). Our experimentally supported model suggests that ligands bind within the upper part of the helical bundle between TM3, TM4, TM5, and TM6. For both linoleate and GW9508, the negatively charged headgroups are oriented toward the extracellular opening of the cavity, whereas the hydrophobic tails, in semi-folded conformation, are oriented toward the center of the receptor. In particular, the carboxylate groups of these ligands are coordinated by three residues: Arg-183(5.39) and Arg-258(7.35), both conserved through the FFAR1 cluster, and Asn-244(6.55). Mutation of these residues markedly impairs activation of FFAR1 by linoleate or GW9508. A potential involvement of the Arg residues was predicted previously by the chemogenomic analysis mentioned above (18). Residues located at position 6.55 have been shown to be important components of ligand recognition in several members of the nucleotide and lipid subfamily, including the nucleotide $P2Y_1$-like receptors that have a basic residue involved in coordination of ligand phosphates at this site (25). Our model also suggests that the hydrophobic portions of the ligands lie in a pocket lined by aromatic and hydrophobic residues that appear to interact more significantly with GW9508 than with linoleate. In particular, two His residues, His-86(3.32) and His-137(4.56), are predicted to interact with GW9508 in a manner dependent on their protonation status. It is worth noting that residues at position 3.32 are virtually at the center of the putative common binding pocket of 7TMRs and participate in ligand recognition in a number of receptors, including the adrenergic receptors that feature at this site an acidic residue involved in coordination of the ligand amines.

Free Fatty Acid Receptors as Therapeutic Targets for Type 2 Diabetes Mellitus

In 2002, >1.2 million Americans were diagnosed with diabetes mellitus, and 90–95% of these were classified as type 2 diabetics (diabetes.niddk.nih.gov/dm/pubs/statistics/#7). The prevalence of this disease is increasing and is projected to reach epidemic proportions.

Islet dysfunction is a pivotal cause of type 2 diabetes mellitus, which is manifest by impaired insulin secretion and increased secretion of glucagon (26). An important pharmacological strategy for treatment of this disease is to stimulate insulin secretion and to reduce glucagon secretion. The increasing knowledge of the physiology of FFARs and their roles in glucose homeostasis suggested that these receptors might be suitable targets for synthetic drug-like compounds (6, 27).

FFAR1 is the best characterized of these receptors and has been shown to play a role in mediating fatty acid effect(s) regulating glucose homeostasis primarily in the β-cell. A number of studies have demonstrated that fatty acids potentiate glucose-stimulated insulin secretion from β-cells by acting on FFAR1 (9, 23, 28–31). Conversely, chronic overstimulation of FFAR1 in experimental animals fed high fat diets or in overnourished humans may be a contributing factor to type 2 diabetes (31). Although it remains controversial whether FFAR1 offers a protective role in glucose metabolism during chronic activation by FFAs, *ffar1*$^{-/-}$ knock-out mice may be protected from high fat diet-induced hepatic steatosis and impaired glucose homeostasis (Ref. 31, but see Ref. 29). Taken together, these studies suggest that FFAR1 ligands could be useful for enhancing insulin secretion in patients with type 2 diabetes mellitus (agonists) or for blocking negative metabolic consequences of chronic overstimulation (antagonists). Because of this therapeutic potential, FFAR1 has been a target of research in the pharmaceutical industry (22, 23, 32–34).

GPR119 has similarly attracted attention because of its expression in the pancreas and demonstrated effects on glucose homeostasis and food intake/body weight (14). A selective agonist for GPR119, PSN632408, suppresses food intake in fat-fed rats and reduces body weight gain and white adipose tissue deposition after oral administration (4). Another GPR119 agonist, AR231453, acts directly on pancreatic β-cells to enhance glucose-dependent insulin release and to improve oral glucose tolerance in wild-type mice but not in GPR119-deficient mice (35).

The intestinal hormones glucose-dependent insulinotropic polypeptide and GLP-1 (glucagon-like peptide-1) play pivotal roles in glucose homeostasis by enhancing glucose-dependent insulin release and maintaining β-cell mass (36, 37). The GLP-1 receptor (a 7TMR belonging to the secretin family that is expressed in pancreatic β-cells) has proven to be a difficult target for identification of small molecule ligands (11). An alternative therapeutic strategy could involve stimulation of GLP-1 secretion from intestinal endocrine cells. Chu *et al.* (13) showed that AR231453 stimulates GLP-1 secretion, and therefore, GPR119 may regulate glucose homeostasis by this mechanism also.

GPR120, which is not expressed in islets, is indirectly involved in regulation of islet function (7) because FFA activation of GPR120 on intestinal endocrine cells causes release of GLP-1 also (5). As GPR120 stimulation increases GLP-1 levels and as GLP-1 has an effect on appetite and feeding, agonists of GPR120 might be beneficial for treatment of diabetes and obesity (6).

High Throughput and *in Silico* Screening Approaches to the Discovery of Synthetic Ligands

Drug discovery endeavors to date have concentrated on GPR119, for which the development of selective agonists has been reported by OSI Pharmaceuticals and Arena Pharmaceuticals (4, 35), and on FFAR1 (22, 23, 32–34). Modulators of GPR119 and FFAR1 have also been the object of numerous patents filed under the Patent Cooperation Treaty.

Studies of FFAR1-ligand interactions showed a pronounced cross-reactivity of FFAR1 with the nuclear receptor peroxisome proliferator-activated receptor-γ. The promiscuity is not limited to endogenous ligands, which in both cases are medium- to long-chain FFAs, but also includes synthetic thiazolidinediones such as the antidiabetic drug rosiglitazone, which activates FFAR1 with low micromolar potency (38).

The quest for selective FFAR1 ligands prompted a number of high throughput screening endeavors followed by lead optimization through classical solution phase synthesis or solid phase combinatorial chemistry. The arylalkyl derivative of the propanoic acid GW9508 was identified by GlaxoSmithKline as a potent FFAR1 agonist with an EC_{50} of ~50 nM, whereas FFAs exhibit potencies in the micromolar range (22, 23, 33). This compound exhibits significant selectivity against peroxisome proliferator-activated receptors, for which it exhibits potencies in the low micromolar range, and does not activate other members of the FFAR1 cluster. However, it activates GPR120 with low micromolar potency, which is comparable with the potency of long-chain FFAs. A selective FFAR1 antagonist (GW1100) was identified also. Notably, the agonist GW9508 features a free aliphatic carboxylic acid and a linear arylalkylic tail, whereas the antagonist GW1100 features an esterified aromatic carboxylic acid and a branched arylalkylic tail. According to our model, these structural differences may be responsible for the antagonistic properties of GW1100. The lack of a negatively charged headgroup in the antagonist may prevent the disruption of the ground state interactions of the residues at positions 5.39, 6.55, and 7.35 that occurs in the case of agonist binding. Moreover, the branched nature of the arylalkylic tail may prevent the ligand from interacting with aromatic residues located in the core of the binding cavity that may be an important component of the activation mechanism.

Similarly, through chemical elaboration of a bromophenyl derivative of a propanoic acid analog that resulted from high throughput screening, Johnson & Johnson identified a novel series of 3-aryl-3-(4-phenoxy)propanoic acid analogs as agonists of FFAR1 with submicromolar potencies and good pharmacokinetic profiles (32). Moreover, a number of β-substituted arylalkylic carboxylic acids have been recently identified by researchers at Amgen (34).

We have used our model of FFAR1 in a virtual screening approach to identify novel ligands for the receptor (39). This *in*

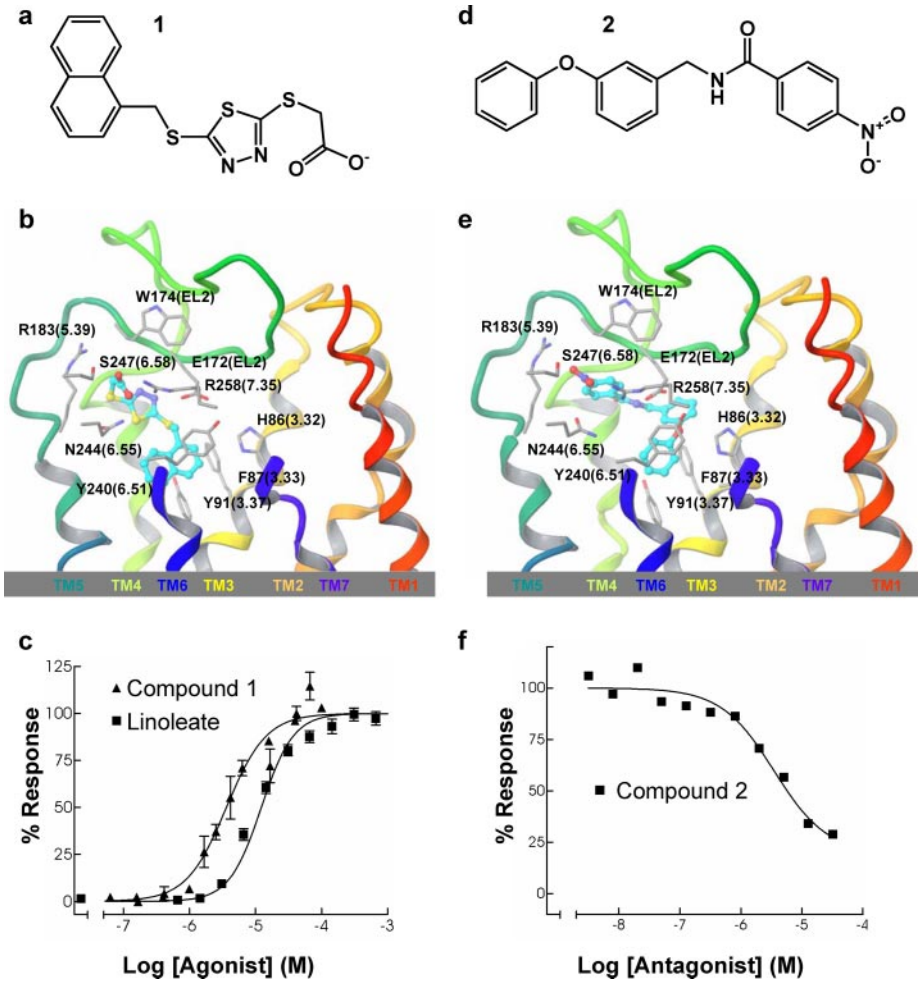

FIGURE 2. **Molecular structures (*a* and *d*), proposed binding modes (*b* and *e*), and dose-response curves (*c* and *f*) of an agonist (*compound 1*) and an antagonist (*compound 2*) of FFAR1 recently retrieved by virtual screening on an experimentally supported homology model of FFAR1.** In the molecular models, the backbone of the receptor is schematically represented as a ribbon colored according to residue position (N terminus (*red*) and C terminus (*violet*)). For clarity, the backbone of the extracellular ends of TM6 and TM7 and that of the third extracellular loop are not represented. The ligands are depicted as balls and sticks, and feature carbon atoms are colored in *cyan*. *c* shows the dose-response curve for the stimulation of FFAR1 by *compound 1* and linoleate. The dose-response curve for the antagonist *compound 2* shown in *f* was obtained by stimulating the receptor with linoleate.

silico approach used the chemical structures of known FFAR1 ligands in combination with the model of the receptor's binding pocket to reveal a diverse set of active compounds in high yield. Specifically, a large data base of commercially available compounds was screened on the basis of (*a*) two-dimensional similarity to GW9508 and another related analog, (*b*) matching to a three-dimensional pharmacophore representing the key groups of the docked conformation of the two ligands, and (*c*) high throughput molecular docking on the FFAR1 model. This procedure led to the identification of six diverse FFAR1 ligands (two full agonists, three partial agonists, and one pure antagonist) out of 52 molecules tested, yielding a hit rate of 12%. Eight additional partial agonists and one additional antagonist were subsequently identified by scanning for close neighbors of the six primary hits. All of the retrieved compounds showed low micromolar potencies; the chemical structures, dose-response curves, and docking complexes of the most potent full agonist (*compound 1*) and the most potent antagonist (*compound 2*) are shown in Fig. 2. As with GW9508, the two full agonists are characterized by an aliphatic carboxylate coordinated by Arg-183(5.39), Asn-244(6.55), and Arg-258(7.35). Conversely, the two antagonists are characterized by the substitution of the carboxylate with a nitro group. Structure-activity relationships suggest that this substitution may be responsible for their antagonistic behaviors.

Future Directions

Patients with type 2 diabetes mellitus experience a progressive deterioration of β-cell function and mass (26). Impaired β-cell function and possibly β-cell mass appear to be reversible, particularly at early stages of the disease, where the limiting threshold for reversibility of decreased β-cell mass has probably not been passed (26). Therefore, the ability to assess residual β-cell mass in patients at risk for type 1 or 2 diabetes would offer significant clinical benefit for evaluating therapeutic strategies. No methodologies for imaging live β-cells *in situ* are currently available. It is conceivable that the highly selective expression of FFAR1 on the surface of β-cells might present a suitable target for imaging approaches, *e.g.* positron emission tomography, using a high affinity ligand. New high potency ligands could also be helpful for development of a radioligand for direct assessment of binding to FFAR1 in *in vitro* studies that would greatly facilitate elucidation of structure-function relationships for this receptor. Therefore, ligands for FFARs could be developed for these purposes, in addition to drugs to treat patients with diabetes mellitus and ligands to explore the biology of FFARs.

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